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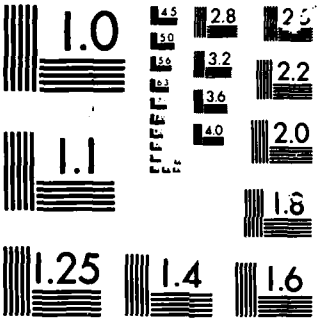
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EFFECTS OF ANTICHOLINESTERASE EXPOSURE ON TRANSPORT AND DISTRIBUTION OF HIGH VS. LOW AFFINITY MUSCARINIC CHOLINERGIC RECEPTORS

Annual Summary Report

James K. Wamsley

November 11, 1983

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Axonal transport of muscarinic cholinergic receptors has been demonstrated in the brain. The receptors in reference emanate from the septum and are transported through the fimbria to the hippocampal formation. These muscarinic receptors represent presynaptic autoreceptors in the cholinergic projection from the medial septum to the hippocampus. The overall density of muscarinic receptors in the hippocampus is only slightly increased after a septal lesion, but this small increase represents a loss of high affinity agonist (see reverse)

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binding sites which is overshadowed by a dramatic increase in the low affinity agonist state of the receptor found in several hippocampal regions. This same shift in muscarinic receptor agonist affinity states is seen in lumbar levels of the cat spinal cord following transection of the cord at cervical levels. Chronically thiamine deficient laboratory rats also exhibit a shift in the agonist state of the muscarinic receptor to that of a lower affinity except in the ventromedial hypothalamus where an overall decrease in density is manifest.

Methodology for direct labeling of high affinity muscarinic agonist receptors has been perfected using the agonist cis methyldioxolane. These sites have been localized autoradiographically. Subtypes of muscarinic antagonist sites have also been localized using a tritiated form of the atypical antagonist pirenzepine. Thus, it will be possible to localize several different muscarinic cholinergic receptor subtypes in animals chronically exposed to anticholinergics or anticholinesterases. It will also be possible to study the effects of this exposure on axonal transport of muscarinic receptors in the brain.

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Abstract

The initial phase of this research project was designed to demonstrate and characterize the phenomenon of axonal transport of muscarinic receptors in the central nervous system. Further, methods were to be developed and perfected for the microscopic localization of subtypes of muscarinic receptors in the brain. These experiments were to be performed in preparation for the second phase of this study. The latter phase involved the determination of how the muscarinic receptor subtypes are affected by chronic exposure to low doses of anticholinergics or anticholinesterases and to determine how the receptor affinities and/or densities can be regulated.

Most of the initial experiments have been completed. The phemonemon of axonal transport of muscarinic receptors into the hippocampal formation was demonstrated by autoradiographic localization of muscarinic receptors in animals with fimbria lesions. This same experiment, performed after a septal lesion or after injection of colchicine into the septum, showed no accumulation of muscarinic receptors. These results indicate that the muscarinic receptors being transported into the hippocampus are coming through the septal projections and likely represent presynaptic autoreceptors on cholinergic neurons.

Research accomplished during the first year of this contract has also provided a means of localizing subtypes of muscarinic receptors. Subtypes of antagonist binding sites have been localized using tritiated quinuclidinyl benzilate and tritiated pirenzepine. These results indicate the presence of two distinct muscarinic receptor subtypes (M_1 and M_2) in the brain. The data suggest that these two antagonist receptor subtypes have high and low affinity agonist site conformations associated with them. These can be labeled indirectly (using carbachol to displace tritiated N methyl scopolamine binding) or directly (using the tritiated muscarinic agonist cis methyldioxolane). The agonist conformations of the M_2 site are affected by the presence of guanine nucleotides while those for the M_1 sites are not. These novel findings indicate the complexity of the muscarinic receptor system. At the same time, these observations support the provocative hypothesis that we may be able to influence the affinity states of the muscarinic receptor in a carefully controlled drug environment. This could have far-reaching ramifications in the many systems in which muscarinic receptor function plays an integral role.

Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH)78-23, Revised 1978).

Publications

Much of the work outlined in the present report is in various stages of publication. Some of the work has been presented at the most recent Society for Neuroscience Meeting in Boston and at the International Conference on Dynamics of Cholinergic Function held in Oglebay, West Virginia.

Preparations for Receipt of Surety Materiel

All of the requirements stipulated by the Chemical Surety Safety Office in preparation for receipt of the chemical surety materiel have now been met as follows: (1) An emergency shower and eyewash station have been installed in the laboratory; (2) a welded stainless steel duct has been installed which runs from the fume hood in the laboratory to a vent on top of the fifth floor and does not interconnect with any other ventilation system within the hospital; and (3) an ultralow -70°C freezer has been delivered for storage of the chemical surety materiel.

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I. Transport of Muscarinic Receptors.

Results obtained from various physiological studies have led to the hypothesis of the existence of presynaptic mechanisms retarding the release of neurotransmitter substances from neuronal terminals. This "presynaptic inhibition" is thought to be receptor-mediated and can result as a consequence of one neuronal terminal impinging on another neuron's terminal (axo-axonic synapse). Presynaptic inhibition can also result from the presence of receptors for a certain neurotransmitter substance strategically located on the terminal membrane of the neurons releasing that particular neurotransmitter substance (autoreceptors). In this way, acetylcholine released into a synaptic cleft can stimulate the postsynaptic membrane via postsynaptic receptors, and at the same time can inhibit further release of acetylcholine by slightly depolarizing the presynaptic membrane via the presynaptic muscarinic receptors.

Studies performed in the peripheral nervous system have indicated that muscarinic receptors are being transported in both the vagus and sciatic nerves (1,2). These transported receptors are thought to represent presynaptic receptors synthesized in the cell bodies (where the components for protein biosynthesis are located) and "shipped" to the terminals where they are incorporated into the terminal membrane. To demonstrate that this same process was taking place in the central nervous system required the identification of a white matter pathway, containing a cholinergic projection, which could be interrupted in order that the transported sites could accumulate. The fimbria of the hippocampal formation containing the cholinergic projection from the medial septum to the hippocampus seemed to fit these requirements precisely.

A microelectrode was stereotaxically lowered into the brain of laboratory rats under ketamine anesthesia. The electrode was positioned in the fimbria just rostral to the hippocampus and a radiofrequency lesion was created using an RGF-4A lesion generator (David-Kopf Instruments, California). The animals were allowed to survive for 24 hours before they were sacrificed by intracardial perfusion with isotonic phosphate buffered saline (pH 7.4). The brains were removed and quickly frozen on dry ice. Ten micron thick sagittal sections were then cut from the brains, using a cryostat, and these sections were thaw-mounted onto microscope slides. Muscarinic receptors were localized on these tissue sections using autoradiographic techniques as described previously (3). Slide mounted tissue sections were incubated for one hour in phosphate buffered saline containing 1 nanomolar tritiated N-methyl scopolamine ([^{3}H]-NMS) followed by two 5-minute rinses in fresh buffer. Control sections were incubated under the same conditions with the added presence of 1 micromolar atropine in the incubation media. After drying, the sections were apposed to sheets of LKB Ultrofilm (LKB Instruments, Rockville, MD) in X-ray cassettes for generation of the autoradiograms.

Examination of the autoradiographic grain distribution (indicating regions of receptor-bound [^{3}H]-NMS) showed an accumulation of receptors had taken place just rostral to the lesion. A smaller accumulation was also seen distal to the lesion site. Therefore, it appeared that the muscarinic receptors were being transported toward the hippocampus through the fimbria and, to a lesser extent, away from the hippocampus (4).

More recently accomplished experiments have allowed the demonstration that these receptors accumulate with time (animals were examined 6,12,18,24, and 48 hours after lesioning), reaching a maximum density between 18 and 24 hours post-lesion. If the septum is electrothermally lesioned 24 hours prior to the fimbria lesion, the accumulation of muscarinic receptors does not occur. Also, if colchicine is microinjected into the medial septum 24 hours prior to the lesion of the fimbria, the accumulation of muscarinic receptors does not occur. Therefore, it would appear that these transported muscarinic cholinergic receptors represent presynaptic autoreceptors on the cholinergic projection from the medial septum to the hippocampus.

Previous autoradiographic studies have provided a means of localizing subtypes of muscarinic agonist sites (3,5). Using this methodology to localize the subtypes of agonist binding sites on the fimbria lesioned sections, it was possible to demonstrate that the high affinity agonist sites accumulate initially. After about 6 hours, the low affinity sites begin to accumulate reaching a maximum at about 24 hours and then falling off at 48 hours post-lesion. There are two possible explanations for these latter findings. One possibility is that the high affinity agonist conformation of the muscarinic receptor is being transported at a faster rate than the low affinity sites and thus accumulate initially. Another possibility is that the high affinity sites accumulate and then are soon altered to their low affinity conformation after several hours post-lesion. Studies are under way to determine which of these two possibilities represents the appropriate case for the transported receptors in the fimbria of the hippocampal formation.

II. Muscarinic Receptor Up-Regulation After Deafferentation.

In a previous study, investigators failed to demonstrate the presence of presynaptic muscarinic receptors within the hippocampus. The study involved the use of receptor binding techniques to localize muscarinic receptors after labeling them with a tritiated form of the muscarinic antagonist quinuclidinyl benzilate ([^{3}H]-QNB). Muscarinic cholinergic receptors were measured in the hippocampus of animals with and without septal lesions. The study (6) was inconclusive since the investigators used only antagonist binding to determine the density of muscarinic receptors. More recent experiments have been performed using autoradiographic techniques to differentiate high from low affinity agonist binding of the muscarinic receptor following septal lesions. The latter method has provided the means for demonstrating that the high affinity agonist sites are reduced while the low affinity agonist sites increase following septal lesions. The latter study (7) also demonstrated that when antagonist binding to the muscarinic receptor was used, little change in the overall density of muscarinic receptors was noted. Thus the reduction in the high affinity agonist site was overshadowed by the increase in the binding to the low affinity agonist site. These results could indicate that the high affinity agonist conformation of the receptor which is transported presynaptically is lost following the septal lesion, while the postsynaptic low affinity agonist conformation is increased due to denervation supersensitivity. This overall shift in binding affinities occurring in the hippocampus following septal lesion remains up to two months post-lesion.

In another study accomplished during the tenure of this contract,

muscarinic receptors in the lumbar spinal cord of cats were examined at various intervals following complete transection of the spinal cord at cervical levels. This study was accomplished in collaboration with John Lane, of the Department of Pharmacology at the Texas College of Osteopathic Medicine in Fort Worth, and with James Smith, of the Department of Psychiatry at the University of Louisiana at Shreveport. This is another case in which the total density of muscarinic receptors was not dramatically changed, but the affinity states of these muscarinic receptors for agonists were greatly altered (7).

Previous studies have demonstrated that the normal conformation of the muscarinic agonist site in the dorsal horn of the spinal cord is the low affinity state, while in the ventral horn of the spinal cord the high affinity agonist state predominates (8). Following cervical transection of the spinal cord, the low affinity sites in the dorsal horn of the spinal cord underwent a 38% alteration to the high affinity state of the muscarinic receptor (7). This alteration reached a peak at two days following transection when there was an increase in the total number of muscarinic receptors. This increase in total binding returned to normal at about 14 days. The shift to the high affinity site of the receptor remained intact until approximately 48 days post-lesion, when the total receptor density, as well as the percentage of high and low affinity agonist states of the receptor, returned to normal. In the ventral horn, however, an increase in the total number of receptors was again manifested at approximately two days following transection. This increase in muscarinic receptor density returned to normal at seven days post-transection. There was a dramatic increase (approximately 35%) in the number of low affinity agonist conformations of the muscarinic receptor in the ventral horn. The increase in low affinity agonist binding sites within the ventral horn was maintained to the longest point examined, in this case, 48 days.

The results from previous studies have indicated that the low affinity agonist state of the muscarinic receptor may be the one associated with the physiological function (9-11). The animals used in the present study manifest a prolonged spasticity due to the lesion of the upper motor neurons. This spasticity may be due in part to the changing of the muscarinic cholinergic receptors present in the ventral horn to their functional conformation on the alpha motor neurons where the low affinity sites are not normally present. This study also demonstrates the fact that the use of simple antagonist binding to demonstrate alterations in muscarinic receptors following perturbation of the central nervous system may not be sufficient to uncover dramatic changes in the affinity states occurring within these central neurons. Thus, the many studies which have attempted to localize changes in muscarinic receptors occurring as a result of human disease states may have been negative due to the fact that the alterations occurred in the affinity states of the receptor rather than a change in overall density of the receptors. I have recently examined the normal distribution of muscarinic receptors in the human cortex (12,13) and I intend to look for differences in these receptors in the cortexes of patients with diseases which have been related to alterations in the cholinergic system.

These findings are in keeping with the investigations of Birdsall et al. (14) and Hulme et al. (15) who demonstrated that classical muscarinic antagonists recognize all of the subtypes of agonist receptors with equal

high affinity. Therefore, using agents such as [^{3}H]-NMS or [^{3}H]-QNB to label muscarinic receptors, only the overall change in the density of these receptors will be defined. It will be important in the remaining tenure of this contract to determine how these subtypes of agonist binding states of the muscarinic receptor are affected by the chronic exposure to anticholinergics or anticholinesterases.

III. Alterations in Muscarinic Receptors Following Chronic Thiamine Deficiency.

Another instance where the importance of localizing high vs. low affinity agonist states of the muscarinic receptor is exemplified in the case of the muscarinic receptor alterations occurring following chronic thiamine deficiency (16). A severe thiamine deficiency is thought to result secondarily to chronic alcohol ingestion. The intensely confusional state the chronic alcoholic presents upon admission to the emergency room can often be rapidly reversed by injection of thiamine. Chronic alcohol abuse has been shown to decrease acetylcholine turnover and decrease acetylcholine biosynthesis without changing the structure of the cholinergic neuron. In a recent study, Gibson et al. (17) demonstrated that the memory loss associated with chronic thiamine deficiency in laboratory rats could be reversed with the cholinergic agonist arecoline or by administration of an anticholinesterase. This recovery of memory in some instances was more efficiently brought about by the administration of the cholinergic compounds than it was by administration of thiamine itself.

To investigate the state of the muscarinic receptor in chronic thiamine deficiency, we developed four groups of animals. Group 1 was maintained on a thiamine deficient diet, while Group 2 was maintained on the same diet with added thiamine. Group 3 was maintained on a diet deficient in thiamine, and was given supplemental injections of thiamine plus the thiamine antagonist pyrathiamine. Group 4 was also maintained on the diet deficient in thiamine and was given only the supplemental injections of thiamine itself. These latter two groups were developed in order to maintain a stable thiamine input independent of caloric intake. Following the development of overt signs of chronic thiamine deficiency, the animals in each group were sacrificed by intracardial perfusion and prepared for muscarinic receptor autoradiography as outlined previously. The alterations in muscarinic receptor densities induced by the chronic thiamine deficiency were examined and quantitated using a DADS Model 560 microdensitometry system (an Oki computer interfaced with an MPV Compact microphotometer attached to a Leitz Orthoplan microscope). Again, in this case, the overall density of muscarinic cholinergic receptors was not altered appreciably due to the chronic thiamine deficiency. However, upon examining the relative concentrations of high vs. low affinity muscarinic agonist binding sites, it appeared that an increase in the low affinity agonist state of the muscarinic receptor had taken place in several regions of the brains from the thiamine deficient animals. Most important, alterations in the density of low affinity sites were found in various regions of the hippocampal formation and in the cortex. This increase in the functional state of the muscarinic receptor within areas known to be involved in memory and consciousness could contribute to the confusional state of the alcoholic and the phenomenon of memory loss in these individuals.

Interestingly, in the same animals, a decrease in the muscarinic receptor density within the ventral medial hypothalamus was noted. This region of the brain is thought to be involved in control of appetite. It is the so-called "satiety" center of the brain. The role of muscarinic receptors in mediating appetite is unknown at this time. The fact that the chronic thiamine deficient animals were extremely anorexic provides some very provocative ideas about the potential role of muscarinic receptors in appetite control. Further studies are currently underway in order to determine how chronic thiamine deficiency effects the transport of muscarinic receptors as well as affects the regulation of conversion from the high affinity to low affinity agonist states of the receptors in these various brain regions.

IV. Localization of Muscarinic Receptor Subtypes.

Other studies accomplished during the first year of this contract included the localization of various subtypes of muscarinic receptors previously unreported. The first demonstration of subtypes of agonist binding sites for the muscarinic receptors using a direct labeling technique was accomplished using a tritiated form of the muscarinic agonist cis methyldioxolane ([^{3}H]-CD). Slide mounted tissue sections of rat brain were prepared as outlined previously and pre-incubated for 20 minutes in 10 millimolar sodium potassium phosphate buffer including 200 millimolar sucrose. The sections were then incubated in the same buffer medium containing a 5 nanomolar concentration of radioactive CD. The incubation was performed for 120 minutes at 0-4°C followed by a 3-second rinse in ice cold buffer alone. Examination of the autoradiograms generated from tissues prepared in this fashion showed highly specific binding of the radioactive muscarinic agonist in several regions of the rat brain previously reported to contain the high affinity agonist conformation of the receptor as defined by indirect labeling techniques. This was the first demonstration of binding of muscarinic agonists to microscopic regions of the brain as defined by direct labeling for receptor autoradiography.

The discovery of the atypical muscarinic antagonist pirenzepine has prompted the definition of subtypes of muscarinic antagonist sites. Binding studies performed in homogenate preparations have indicated that [^{3}H]-QNB labels sites in addition to those that can be labeled with [^{3}H]-pirenzepine ([^{3}H]-PZ). In order to localize these subtypes microscopically, we labeled serial sections of rat brain with either [^{3}H]-PZ (18,19) or [^{3}H]-QNB (5,20). By comparing the autoradiograms generated by sections treated in this fashion, we were able to define a subpopulation of muscarinic receptors recognized by [^{3}H]-QNB but not labeled with [^{3}H]-PZ.

Using the classification of Goyal and Rattan (21), we would define the sites labeled with [^{3}H]-PZ as M_1 receptors, whereas those that were labeled with [^{3}H]-QNB and not labeled with [^{3}H]-PZ represent M_2 receptor sites. This study thus presented the first information on the differential localization of M_1 vs. M_2 sites in discrete regions of the brain. Interestingly, the sites defined as M_2 sites, that is, the sites labeled with [^{3}H]-QNB and not labeled with [^{3}H]-PZ, were invariably found in regions of the brain previously shown to have high concentrations of the high affinity agonist state of the receptor as defined by indirect labeling techniques (3,8) and by

direct labeling with the tritiated agonist [^{3}H]-CD (22). Thus, the M_1 sites labeled with pirenzepine represent areas where the low affinity agonist conformation of the muscarinic receptor exists. This observation raised the possibility that the low affinity agonist state of the receptor could be the M_1 binding site. In order to examine this, we attempted to convert the high affinity agonist state of the receptor to the low affinity agonist state using guanine nucleotides in the incubation medium (23-25).

Previous studies have demonstrated that in the presence of guanosine triphosphate (GTP), the high affinity agonist state of the cardiac muscarinic receptor can be converted to a low affinity agonist state (26-29). This has been demonstrated autoradiographically on the population of orthogradely transported muscarinic receptors of the peripheral nervous system (2). In the present study, it was determined that the same guanine nucleotide regulation occurs within the muscarinic receptors found in the spinal cord. By including a 10 micromolar concentration of guanyl-5'-ylimidodiphosphate (GppNHp), a nonhydrolyzable analog of GTP, in the incubation medium, it was possible to convert the high affinity agonist sites in the ventral horn to their low affinity conformation. [^{3}H]-PZ sites are not normally found in the ventral horn of the spinal cord, and with the inclusion of GppNHp in the incubation medium, no increase in [^{3}H]-PZ binding was found in the ventral horn (7). It therefore appears that under these conditions, the [^{3}H]-PZ sites are not increased in an area where the conversion of high affinity agonist sites to low affinity agonist sites has been accomplished. The data, rather, fit the hypothesis that two non-interconvertible muscarinic receptors (M_1 and M_2 sites) exist within the brain (30-32). Both of these subtypes of muscarinic receptors may have convertible high and low affinity agonist sites associated with them. Both indirect and direct labeling techniques which define the high affinity agonist site preferentially label this conformation of the M_2 receptor subtype.

Previously mentioned studies have shown that the high affinity agonist sites can be converted to the low affinity agonist state using guanine nucleotides. The mechanisms underlying the conversion of high affinity M_1 sites to a lower affinity state are not as well defined, but these receptors may be under the regulation of such compounds as N-ethylmaleimide (33). Further studies will be necessary to better understand the regulation of these muscarinic receptor subtypes. The information at hand, however, will be important and useful in determining the effects of chronic anticholinergic or anticholinesterase exposure on muscarinic receptors within the brain.

With the advent of the methodology for labeling M_1 vs. M_2 muscarinic receptor subtypes using [^{3}H]-PZ and [^{3}H]-QNB, it was possible to define which of these muscarinic receptor subtypes was being transported in the brain and in the peripheral nervous system. Replication of the ligature experiments performed on the vagus and sciatic nerves (1,2) resulted in the demonstration of muscarinic cholinergic receptors transported in both orthograde and retrograde directions which could be labeled with either [^{3}H]-QNB or [^{3}H]-PZ. These two muscarinic antagonists also equally labeled the muscarinic receptor sites accumulating on either side of a lesion placed in the fimbria of the hippocampal formation (7). Thus, in these systems it appears that it is the M_1 receptor subtype which is being transported. The high affinity agonist sites which accumulate adjacent to these lesions apparently represent the high affinity agonist conformation of the M_1 receptor.

The selective muscarinic antagonist pirenzepine could produce some interesting behavioral changes in the central nervous system not shared by the classical antagonists such as atropine or QNB. However, since pirenzepine does not readily cross the blood/brain barrier it is difficult, at this time, to study what these differences may entail. Pirenzepine does show fewer muscarinic cholinergic side effects when administered peripherally for the treatment of peptic ulcers. For instance, pirenzepine does not readily cause such muscarinic side effects as tachycardia and dry mouth, which are seen with other muscarinic antagonists. Pirenzepine is capable of reducing gastric acid secretion, although the exact level of this effect is not known. As part of the research accomplished under this contract, it has been possible to show labeling of muscarinic cholinergic receptors in the human stellate ganglia with both [^{3}H]-QNB and [^{3}H]-PZ (34,35). We are currently attempting to demonstrate that tritiated pirenzepine also labels sites within the intramural ganglia of the parasympathetic nervous system lining the gut. Pirenzepine has been shown to be a weak inhibitor of acid secretion in isolated rat parietal cells (36) which would support the hypothesis that pirenzepine's antisecretory activity may be attributable to some mechanism within the peripheral ganglia.

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